Highlights from the Seventh International Workshop on HIV Persistence during Therapy, 8–11 December 2015, Miami, Florida, USA

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Abstract

Over 4 days, more than 270 scientists involved in HIV persistence research convened to share their data and discuss future avenues to control HIV without continuous antiretroviral therapy.

This 7th International Workshop on HIV Persistence followed the format of the preceding conferences but more time was given for discussing abstracts submitted by the participants and selected by the Steering and Scientific Committees.

The topic of the workshop is HIV persistence: consequently, issues of HIV reservoirs and HIV cure are also addressed. In this article we report as closely as possible what was discussed. However, owing to length constraints, not everything is reported here but all the Workshop abstracts can be found online (www.viruseradication.com).

Keywords: HIV persistence, HIV reservoirs, HIV cure, HIV functional cure, HIV eradication

Session 1: *in vitro* and *in vivo* models of HIV persistence

This is a vexing but important area in need of further development, as the complex and combinatorial approaches that will be needed to fully clear established HIV infection require robust, flexible, and reproducible model systems that can reflect the complexity of persistent HIV infection to the fullest extent possible.

J. Victor Garcia [1] opened the session with a plenary discussion of his group's systemic examination of the latent and residual active HIV reservoirs in the bone marrow-liver-thymus (BLT) humanised mouse model, following HIV infection and full combined antiretroviral therapy (cART) suppression. Dr Garcia reviewed the broad dissemination of resting CD4 T cells in tissues throughout the mouse model containing truly latent, replication-competent HIV that could be induced to grow following *in vitro* activation. Tissues examined also exhibited low-level viral RNA expression, indicating the presence of what is termed the 'residual active HIV reservoir,' also systemic in nature. The BLT model was shown to allow the evaluation of the effect of a broad array of pharmacological and biological interventions on persistent infection. Dr Garcia closed with a description of the MoM, or myeloid-only mouse, a new model in which the mouse is reconstituted with B cells and macrophages. The MoM was infected by HIV strains that can infect macrophages, durable viraemia was supported in the model, and fully suppressed by cART in the plasma, with (as in the BLT) residual HIV-RNA expression measured in the tissues despite cART.

Stephen Hughes [2] reported the study of clonal amplification of SIV-infected cells in the spleen and lymph nodes of rhesus macaques. In parallel to recent work in humans [3,4], after SIV infection and 1 year of suppressive cART, approximately 380 independent integration sites were identified in cells from tissue, and 13 of these were represented multiple times, identifying 13 clones of expanded cells. Two of the clones were present in both the spleen and lymph node. It remained to be determined, as in humans, if these expanded HIV genomic clones were always, sometimes, or never capable of producing replication-competent virus.

Matt Marsden [5] discussed the ability of synthetic compounds based on the natural protein kinase C agonist molecules prostratin and bryostatin to reverse HIV latency in the BLT mouse model. The compounds strongly upregulated CD69 expression on both CD4+ and CD4- T cells in the blood, but on very few cells in tissue. It is not clear if broad CD69 activation is a desirable quality. The bryostatin analog SWU034 upregulated the expression of an HIV strain marked by an antigen tag to a variable extent (from no upregulation up to 7-fold increase) in several animals *in vivo*; a variable dose-response was also seen.

Woelk and White [6] suggested that transcriptomics could be used to assess model validity and to identify biomarkers of latency. Using a primary central memory CD4 T cell (T_{CM}) model of HIV latency, they found 827 differentially expressed genes in latently infected cells, associated with processes such as p53 signalling and DNA damage. Comparison with similar data from a different cell model identified an overlap of 51 upregulated and 32 downregulated genes. It is hoped that such work will ultimately identify gene profiles of latent infection relevant to cells from HIV-infected patients.

Anna Aldovini [7] found that the addition of a p38 MAPK inhibitor to cART in the SIV model did not further reduce the residual viraemia in blood, or negatively affect anti-SIV immune responses, and was well tolerated. However, numerous markers of immune activation were significantly reduced compared to the cART-alone group, suggesting that the p38 MAPK inhibitor used in this study, which is already in clinical trials for other inflammatory diseases, could help reduce immune activation not fully reversed by cART in humans.

Deanna Kulpa [8] generated central, transitional and effector memory CD4+ T cell subsets *in vitro* following HIV infection and cytokine treatment in the presence of cART to prevent viral replication and spread in culture. When established by this 10-day

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protocol, the central memory (T_{CM}), transitional memory (T_{TM}), and effector memory (T_{EM}) populations were differentially sensitive to various latency-reversing agents (LRAs). The T_{CM} population was more resistant to most LRAs, but was more sensitive to some agents than T_{TM} or T_{EM}. The work could be viewed in several ways: emphasising the need to focus on central memory latency, or to seek specialised strategies for each potential pool of latently infected cells. This finding was later echoed by Beliakova-Bethell [9] in the second session, who found that histone deacetylase (HDAC) inhibitors (HDACi) exhibit both inducing and inhibitory effects on HIV reactivation in CD4+ T cells, but that they may be most active in naïve cells, the minority component of the latent reservoir. However, it could also be pointed out that in cells from patients, true latency was not found in T_{EM} cells, only rarely in T_{TM} cells, and uniformly in T_{CM} cells [10].

Hans Peter Kiem [11] reported the impact of autologous transplantation on viral reservoirs in a non-human primate model of HIV/AIDS. Animals were infected with HIV enveloped/SIV chimera viruses (SHIV), and suppressed by three-drug ART. For autologous transplantation, myeloablative total body irradiation is used to increase engraftment of subsequently infused haematopoietic stem cells (HSCs), and to target latently infected cells. However, transplant led to more severe rebound plasma viraemia following cART withdrawal, with higher levels of viral RNA in tissues during cART, despite well-suppressed plasma viraemia. The mechanism of transplant-dependent reservoir expansion was unclear.

Session 2: basic science of HIV latency

Jon Karn [12] opened the session with an overview of the mechanisms that establish and enforce latent proviral infection, with many observations contributed by his group. He discussed the multiple epigenetic silencing complexes that may contribute to HIV latency, and highlighted the evidence for the role of the Polycomb complexes, PRC-1 and PRC-2. Good evidence was shown that PRC-2 is required to both establish and maintain HIV silencing, and that the trimethyl mark at histone K27 contributes to viral quiescence, as its removal by EZH2 inhibitors can contribute to latency reversal. The trimethyl histone mark at K9, however, appears primarily important in the establishment of latency, and may therefore be a poor target for LRAs. Other factors such as JARID2 may play a role in the recruitment of PRC to the HIV LTR, and this event may require the participation of a long non-coding RNA. As the definition of the roles that such factors play becomes clearer, new targets or concepts for latency reversal may emerge.

Angela Ciuffi [13] used a single-cell RNA-Seq approach to investigate cellular heterogeneity in HIV infection, seeking to identify biomarkers of HIV permissiveness. She found transcriptional heterogeneity in both highly and poorly HIV permissive donor cells. She proposed that the single-cell-based approach developed in this study could help characterise the heterogeneity of the HIV latent reservoir, as well as the response of latently infected cells to reactivating agents.

Anthony Cillo [14] isolated peripheral blood mononuclear cells (PBMC) from leukapheresis product from donors on suppressive cART for ≥1 year. He quantified cellular unspliced HIV-RNA (CA-RNA) and proviral HIV-DNA (CA-DNA) in uncultured PBMC. Resting CD4+ T (rCD4) cells were then isolated by negative selection, and activated with PMA/ionomycin in the presence of efavirenz. In 22 donors levels of HIV-1-RNA after treatment with PMA/ionomycin varied >1000 fold but CA-RNA and CA-DNA levels were strongly correlated with each other (rho=0.80, P<0.001), and with inducible virion production (rho=0.76, P<0.001 for CA-RNA; rho=0.75, P<0.001 for CA-DNA). He suggested that simple measures of CA-RNA/DNA can be used to estimate the size of the inducible reservoir.

Fabio Romerio [15] presented his findings suggesting that an HIV-1 antisense transcript (AST) is an inducer of viral latency. He suggested that HIV-1 encodes for its own non-coding RNA, an AST as an autonomous mechanism to recruit PRC-2 to the 5′ LTR, and establish latency. He showed that expression of AST suppressed HIV-1 replication, and promoted the establishment and maintenance of latency. This correlated with decreased levels of RNA polymerase II, and increased levels of H3K27me3 and HDAC at the 5′ LTR. Some evidence of direct interaction between AST and PRC-2 was also found. Interestingly, instead of suggesting that AST should be targeted to disrupt latency, Romerio suggested that therapies aimed at stabilising latency and enforcing permanent viral quiescence could be pursued by exploiting AST.

Celsa Spina [16] closed the session with a description of studies in primary cell models of infection and latency establishment. Cells were infected at different times, before or after stimulation by anti-CD3/CD28, and followed out to 14 days when quiescence was re-established. Cell proliferation of acutely infected cultures was tracked using CFSE dye and cell subsets that had undergone varying degrees of proliferation were isolated by FACS at the end of culture. Each recovered cell subset was analysed for the quantity of integrated HIV-DNA and replication-competent virus. Cells exposed to HIV, prior to stimulation, contained the highest levels of integrated provirus and replication-competent virus after returning to quiescence. Cells infected at the height of cell proliferation retained the least HIV. Cells that did not divide or divided only a few times contained higher levels of integrated and replication-competent HIV. Spina concluded that HIV latency is established very early after infection within a heterogeneous population of CD4+ T cells, which are undergoing varying degrees of cell activation in the presence of infectious virus. Minimally activated cells within this milieu are the most likely to develop persistent latent infection. These observations are consistent with the kinetics of latency establishment observed *in vivo* following acute HIV infection [17].

Session 3: virology of HIV persistence

John Coffin [18] opened this session reviewing the current knowledge on the dynamics of HIV nucleic acids in response to cART, underscoring that the decay of cell-associated viral DNA (CAD) is much less profound than that of plasma HIV-1-RNA (Figure 1). A similar picture of significant stability of CAD emerged from studies of viral breakthrough in pre-exposure cART prophylaxis protocols in macaques experimentally infected with SHIV, as presented by J. Gerardo García-Lerma [19].

A pattern of preferred integration sites emerged in PBMC isolated from infected individuals, for example in *MKL2* and *BACH-2* genes, with all proviruses in the same orientation (unlike what is observed upon *in vitro* infection of PBMC or Hela cells), Coffin continued. Furthermore, the common belief that chronically infected cells *in vivo* would carry only defective proviruses is challenged by the example of cells infected with the AMBI-1 provirus (estimated to be 8 million), responsible for the predominant virus in a patient's plasma and confirmed to be replication-competent by a standard quantitative virus outgrowth assay (QVOA). At autopsy, the AMBI-1 provirus was found broadly present in several organs and tissue, including the cancer metastases responsible for the patient's death (as also discussed by Rebecca Rose in the next session). A similar conclusion was drawn by Mary F. Kearney [20] who described the presence of replication-competent genomes as well as of defective proviruses in expanded CD4+ T cell clones isolated from infected individuals. Her approach was supported by single

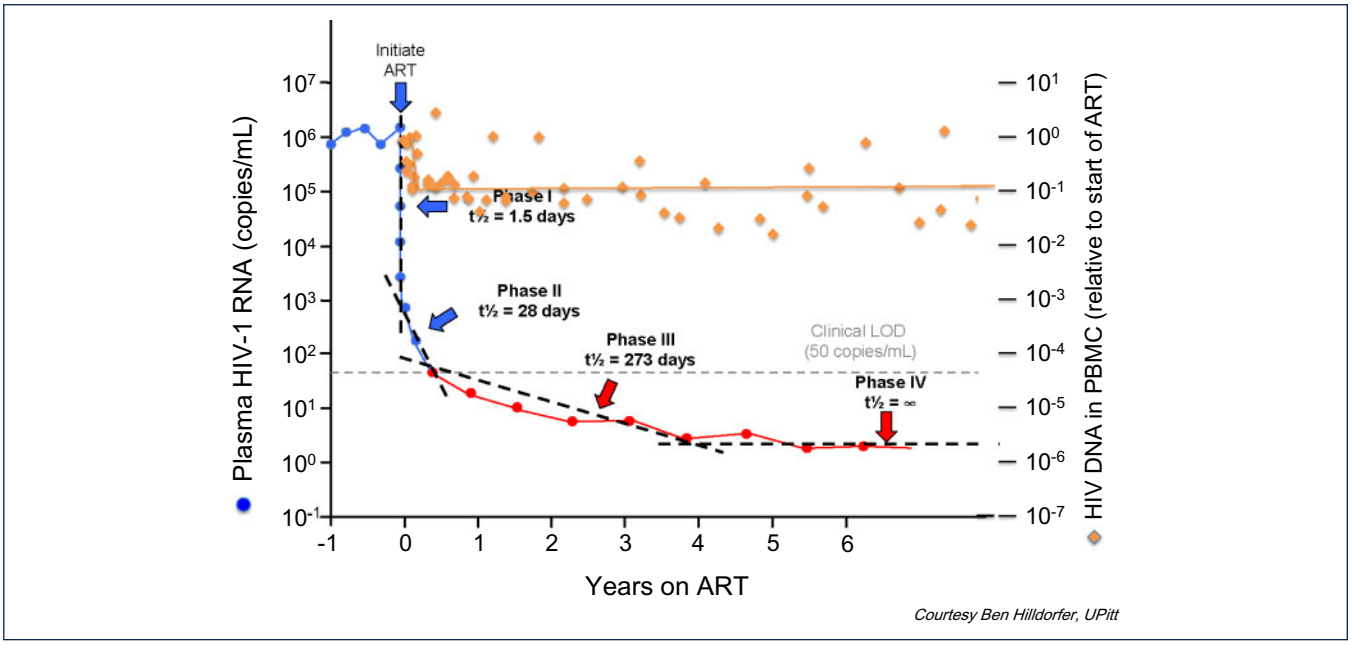

Figure 1. Comparison of HIV-RNA and DNA decay curves upon introduction of cART, as discussed by John Coffin [18]. HIV-1 DNA decays much less than RNA after initiating cART

genome sequencing (SGS) of cell-associated RNA (CAR) in PBMC of infected individuals and it was based on the assumption that different genomes would mark distinct infected cells, whereas identical sequences would be associated with the same cells that eventually underwent clonal expansion. Dr Kearney described 19 identical proviral sequences with intact *pro–pol* genes, four of which were expressing unspliced (US) RNA, which were identified in a single patient. Overall, Dr Kearney reported that at least 13% (range 5–21%) of proviruses expressed US RNA; however, when a standard QVOA was performed to define the fraction of replication-competent proviruses only 0.3% of the expressed proviruses matched the QVOA results, suggesting that the great majority of cells expressing US RNA are indeed carrying defective proviruses. On the same topic, Jason Hataye [21] described that a sustained HIV release of 2400 copies of HIV-RNA/cell/day for 3–6 days post-infection occurred from infected cells once isolated *ex vivo* upon their stimulation with anti-CD3/anti-CD28 monoclonal antibodies (mAbs) in the presence of efavirenz (to prevent virus spreading to newly infected cells).

Searching for novel assays allowing a more accurate quantification of HIV expression upon protocols of proviral reactivation, Bonnie J. Howell [22] described a novel ultrasensitive p24 Gag assay based on a fully automated platform (Quanterix Simoa technology) capable of detecting as low as 3 pg/mL of viral protein with a dynamic range of >4 $\log_{10}$ and enabled to detect the viral protein in cell lysates of cells infected with different HIV clades. On the same issue, Fabio Romerio described a new technology, termed PrimeFlow RNA, based on three HIV-1 probe sets capable of detecting all classes of viral transcripts and allowing the detection of as low as one infected cell out of 10^4 to 10^5 (which is comparable to the number of infected cells observed in peripheral blood of individuals receiving cART) either spontaneously releasing virions or inducible by LRAs [23].

Session 4: anatomic and non-CD4 cell reservoirs

Donna Farber [24] delivered an elegant lecture on the recent concept of tissue localisation of human T lymphocyte responses. Unlike what is commonly believed, i.e. that T cells recirculate freely through tissues and organs in order to patrol the body and to become activated in case of detection of the presence of pathogens, Dr Farber provided convincing evidence of residency

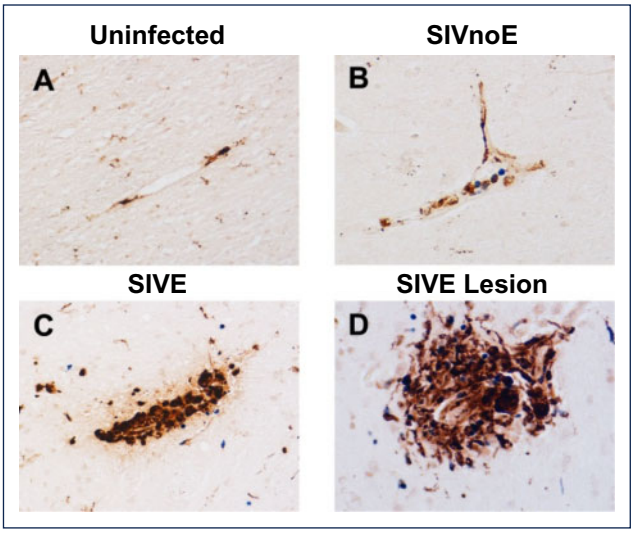

Figure 2. Nuclear Ki-67 IR was restricted to macrophages present in perivascular spaces and meninges. Double-label immunohistochemistry for Ki-67 (blue) and CD68 (brown) in the brains of uninfected and SIV-infected macaques with or without encephalitis shows the presence of 'cycling' macrophages in the perivascular space and within the encephalitic lesions. After Woong-Ki Kim [25]

of significant subsets of T lymphocytes, defined as 'tissue resident memory (TRM) cells'. TRM cells were identified first in the mouse and then in humans and are phenotypically characterised as CD45RA-/CCR7-/CD69+ T cells endowed with the capacity of reacting to tissue perturbation. This general concept has been largely assumed thus far for resident macrophages, but not for cells of the adaptive immune system such as T cells.

Another immunological dogma, i.e. that tissue resident macrophages do not proliferate, was challenged by Woong-Ki Kim [25] in relationship to the pathogenic role of perivascular macrophages (PVM) during SIV encephalitis (SIV-E). By investigating the expression of cell-cycle associated markers, such as nuclear Ki-67, in the meninges and in the perivascular space of the blood–brain barrier, Dr Kim described that the only cells expressing these markers indeed belonged to PVM, as confirmed by their incorporation of a labelled thymidine analog (Figure 2). Of note is the fact that PVM, as previously reported, were also productively infected by SIV. In contrast, the infiltration of

peripheral blood-derived monocytes, labelled by MAC387, a mAb directed against calprotectin, occurs only late in the course of viral infection and seems to play a protective role on the severity of SIV-E. Overall, concluded Dr Kim, these results support a role of PVM as long-term SIV reservoirs, also in consideration of their proliferative capacity.

The importance of lung alveolar macrophage (LAM) infection was discussed by David Gludish [26]. By applying a FISH:FACS combined assay it was possible to identify productively infected cells in the broncho-alveolar lavage (BAL) of infected individuals followed by the Queen Elizabeth Central Hospital in Blantyre, Malawi. The resulting virus turned out to be infectious in TZM-bl cells, as detected by flow cytometry and conventional QVOA, and by co-cultures of LAM from infected individuals and TZM-bl cells, forming elegant foci of infection that could be easily visualised and quantified. Of note is the fact that infectious virus was also rescued by LAM of individuals under cART. The role of LAM in HIV infection was also highlighted by Francis Hong [27] who underscored that while these cells are clearly infected *in vivo* in viraemic patients, their role in those taking cART still remains elusive. In order to further explore this aspect, he conducted a cross-sectional study on 23 infected individuals on effective cART. He detected HIV-DNA and HIV-RNA in 16/23 and in 8/23 individuals, respectively. LAM were then purified from the BAL of either viraemic individuals (n=2) or of individuals taking cART for at least 2 years (n=7). When the levels of HIV-RNA were compared in BAL versus plasma of the same individuals they were significantly lower. In contrast, the levels of CAD in PBMC and LAM were very similar. The possibility that LAM-associated HIV-RNA was consequent to the phagocytosis of infected T cells was ruled out by amplification of T cell receptor genes that tested positive only in one case. It remains, however, to be established whether LAM-associated HIV-DNA is either replication competent or defective and whether its expression is inducible by LRAs.

Rebecca Rose [28] discussed the potential role of lymphatics and cancer tissues as potential reservoirs of HIV-infected cells in patients receiving cART. She investigated post-mortem tissues from 20 HIV-1-positive individuals on cART who had died as a result of different types of cancer, such as plasmacytoma, lymphomas, lung, prostate and anal cancers. Dr Rose noted that expansion of individual proviruses, as identified by SGS, was temporally related to the clinical emergence of cancer metastases. HIV-RNA was found in CNS-associated macrophages and in plasmacytoma. There was also a significant genetic diversity of HIV sequences suggesting ongoing virus replication in spite of the fact that the patients were receiving cART. Thus, Dr Rose concluded, the tumour-associated microenvironment may provide an ideal pabulum for HIV replication and/or proliferation of infected cells.

A classical immunological sanctuary is represented by the human testis, as discussed by Jean-Pierre Routy [29] and the persistence of SIV in this anatomical site has been recently revealed by immuno-PET studies targeting gp120 Env by Ab-targeted positron emission tomography that highlighted an unexpectedly large amount of virus in the testis. For this reason, the Orchid study was launched taking advantage of tissue from adult males choosing to undergo gender reassignment surgery and stopping oestrogen consumption at least 4 weeks before the operation. The presence of CD39+ T regulatory cells secreting the immunosuppressive cytokine IL-10 was observed in the tissue [30]. By immunobeads sorting for CD3 it was also observed that numbers of CD4+ cells were lower whereas CD8+ cells were superior in number in comparison to peripheral blood. In the case of HIV-1-positive donors, these cells were highly activated showing an increased expression of indolamine 2,3 dioxygenase (IDO)-1 and IDO-2 along with increased levels of CD73+ memory CD8+ T cells, therefore contributing to create a 'safe' environment for HIV-infected cells, Professor Routy concluded.

Session 5: immunology of HIV persistence

In an overview, Nilu Goonetilleke (University of North Carolina, USA) listed several challenges associated with the use of HIV-specific T cells in curative strategies including their low frequencies, the rare number of reservoir target cells, the existence of immune privileged sites (like B cell follicles) and escape mutations, and the fact that LRAs may compromise host immunity. Although HIV-specific T cells are rare in durably suppressed adults on cART, they can be detected and their frequency can be measured, suggesting that they may be an important player in curative strategies. However, the number of HIV-specific T cells may be insufficient to clear or control the viral reservoirs. Several strategies can be used to enhance clearance of latently infected cells: the use of chimeric antigen receptors (CARs), dual-affinity re-targeting (DART) or other immune effectors would facilitate the elimination of reactivated cells. A complementary approach would be to disrupt the B follicle in order to access to this cryptic reservoir. In addition, the use of immune checkpoint blockers may improve pre-existing (and also newly induced) T cell functions. Finally, Dr Goonetilleke insisted on the importance of examining LRA effects on HIV-specific T cell functions.

Julia G. Prado [31] compared the level of viral reactivation in CD4+ T cells isolated from HIV controllers and virally suppressed individuals on cART. HIV controllers showed low frequencies of HIV-infected cells, which were associated with inefficient reactivation by different LRAs. Controllers whose CD4+ T cells failed to produce HIV *in vitro* had low HIV-specific CD8+ T cell responses, suggesting that inefficient viral reactivation may suffice to maintain control of infection in the absence of cART.

Genevieve Clutton [32] measured the effects of well-characterised LRAs (including HDACi and protein kinase C-alpha [PKC-α]) on total and HIV-specific T cell functions. HDACi had moderate effects on the pool of total T cells, whereas PKC-α had sustained non-specific effects on all T cells. While vorinostat and ingenol had no impact on the proliferation of HIV specific CD8+ T cells, romidepsin, panobinostat, prostratin and bryostatin all markedly inhibited HIV-specific T cell proliferation. Of note, the effects on antigen-specific CD8+ T cells were time and function-dependent, indicating that a careful evaluation of the impact of each LRA on immune function is warranted before testing these agents *in vivo*.

Alba Ruiz de Andrés [33] presented a novel assay to measure the ability of different LRAs at inducing killing of latently infected cells by CD8+ T cells. The co-culture system uses infected immortalised CD4+ T cells (U937) and HLA-matched CD8+ T cells. In this model, LRAs induced the production of HIV proteins that led to HLA class I antigen presentation and CTL activation. The authors observed differences in T cell receptor (TCR) avidity between donors in response to LRA activation. Importantly, this recognition induced CTL killing.

Vanessa Sue Wacleche [34] presented the results from two studies aimed at identifying new cellular reservoirs for HIV. She first described a novel subset of Th17 cells (CCR6+, CCR4-, CXCR3-referred to as CCR6+ double negative [DN] cells). These cells expressed transcripts associated with early Th17 development (STAT3), follicular helper cells (BCL6), and self-renewal (Lef1 and Terc). CCR6+ DN, similar to Th17 and Th1Th17, proliferated in response to *C. albicans* but not CMV. In contrast to Th17, Th1Th17 and DP CCR6+ that were all depleted in HIV-infected individuals on cART, the frequency of CCR6+ DN was preserved. Surprisingly, all Th17 subsets including the CCR6+ DN carried integrated

HIV-DNA during cART. Importantly, DN CCR6+ cells produced viral particles *in vitro* after stimulation, suggesting that they represent a reservoir for HIV in virally suppressed individuals. As revealed by flow cytometry following TCR activation of these cells *in vitro*, the DN CCR6+ co-expressed Th17 and the p24 viral antigen, indicating that this subset supports HIV replication. In her second presentation [35], Ms Wacleche presented evidence that the transcriptional program governed by RORγt favours HIV-1 replication in Th17 cells. She demonstrated that HIV permissiveness in Th17 versus Th1 is regulated by entry and post-entry mechanisms. Compared to Th1 cells, Th17 cells displayed increased levels of transcripts associated with T cell polarisation/differentiation, circadian clock, TCR signalling, apoptosis, and HIV replication. They also exhibited superior sensitivity to TCR triggering, increased proliferation potential, and superior NF-κB DNA-binding activity. Using small interfering RNA, RORC was shown to be a major positive regulator of HIV replication in Th17 cells.

Eli Boritz [36] analysed viral sequences in T cell subsets to understand the mechanisms responsible for HIV persistence in controllers. He observed that the transitional and effector memory CD4+ T cells account for most infected cells in blood of controllers and that effector memory cells displayed a lower viral diversity when compared to other subsets. This was attributed to the expansion of some T cell clones through proliferation. Interestingly, some cells from these expanded clones were able to produce viral particles in response to TCR stimulation, suggesting that expanded proviruses can carry inducible HIV in controllers. Analysis of viral sequences from blood and lymph node revealed that these two compartments represent distinct reservoirs in HIV controllers.

Julie Boucau [37] closed the session by presenting the effect of LRAs on antigen processing in primary CD4+ T cells. Using a sophisticated system, she showed that resting and activated CD4+ T cells process antigen in different ways. Importantly, HDACi and PKC-α modified cellular protease hydrolytic activities and cause changes in antigen degradation. She highlighted that different *in vitro* systems of HIV latency display distinct peptidase activities, which may result in differences in antigen processing and presentation. This may lead to the presentation of different epitopes at the cell surface of the reactivated cells upon exposure to different LRAs.

Session 6: pharmacology of HIV persistence

Courtney Fletcher [38] highlighted the key issue of ARV penetration and distribution in tissues to efficiently target SIV/HIV persistence in the entire body, including anatomical reservoirs. He first showed that the physical characteristics of ARVs had an impact on the delivery of the active drug that may influence the capacity of each drug to reach an efficient concentration. In addition, the presence of drug transporters on target cells as well as fibrosis in lymphoid tissues also modulated the drug concentration in tissues. Intensive research is currently ongoing to try to modulate each of these factors to improve drug penetration, distribution and consequently efficacy.

More specifically, Reina Bendayan [39] reported on the role of drug transporters and metabolic enzymes in ARV disposition in testicular tissue. She highlighted the fact that persistence of HIV in this particular anatomical sanctuary still needs to be demonstrated. HIV persistence in tissues not only raises the question of ARV pharmacology but also curative strategy efficiency in tissue reservoirs.

Warner C. Greene [40] presented evidence demonstrating that CD4+ T cells isolated from lymphoid tissues are more responsive to LRAs than cells isolated from peripheral blood. His findings warned for more careful testing of eradication strategies in tissues.

John K. Bui [41] presented the results of an extensive analysis of the production and replication competency of *in vitro* expanded clones of HIV proviruses. His findings challenge the dogma of the cytotoxicity of viral production induced by LRAs and the defectiveness of expanded clones.

Session 7: drug discovery

In the era of the HIV 'flush and kill' strategy (Figure 3), it becomes more and more evident that HDACi alone cannot unravel the blockade of HIV expression. Current research focuses on the identification of compounds that potentially act synergistically with HDACi to induce HIV transcription. Farnesyltransferase inhibitors for instance, enhance HDACi effectiveness in cell lines, in primary T cells (J. Karn, unpublished data), as well as in *ex vivo* studies of HIV-infected patient cells (N. Archin and D.M. Margolis, unpublished data). Furthermore, HDACi-treated cells are rendered susceptible to effector cells/'kill' agents and clearance in a primary T cell model of HIV latency *in vitro*. Since HIV infection resides in many different T cell subsets, it is still to be demonstrated that HIV expression induced by an HDACi/LRA association is able to reach relevant reservoirs. Current HDACi in clinical development have multiple liabilities and therefore need improvement concerning potency, specificity of effects and safety as highlighted by Richard Barnard from Merck [42].

Dale Ando presented results from Sangamo BioSciences on the use of zinc finger nuclease technology in CCR5 gene editing in CD4 T cells of HIV-infected subjects [43]. Current studies aim to increase engraftment of autologous CCR5 disrupted CD4 T cells (SB-728-T). The SB-728-1101 Cytoxan (cyclophosphamide or CTX) study is an open-label multicentre study in 18 aviraemic cART-treated HIV subjects, using a dose escalation of IV CTX followed by a single infusion of SB-728-T and a 16-week antiretroviral

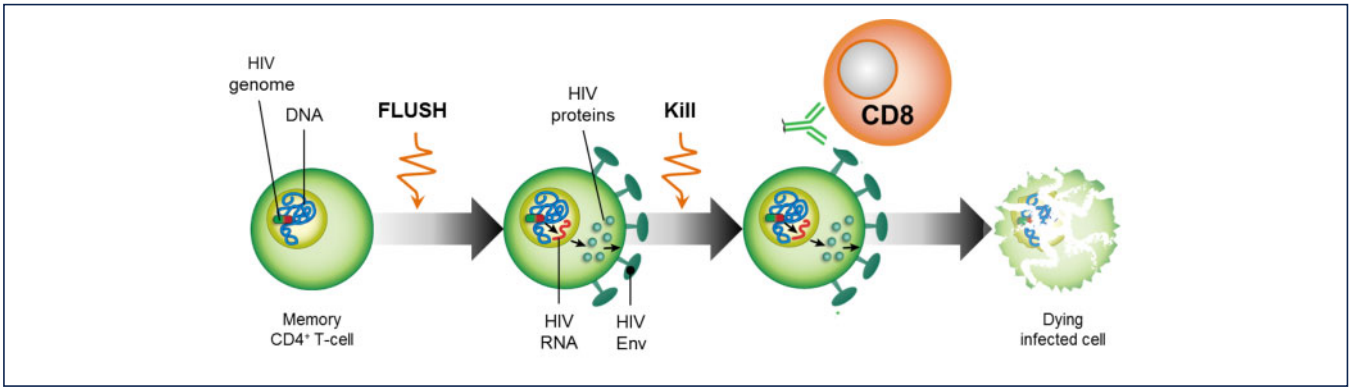

Figure 3. The 'flush and kill' strategy. After Richard Barnard [42]

treatment interruption (TI), beginning 6 weeks after infusion of cells. CTX conditioning at doses up to 1 g/m^2 increases total and CCR5-modified CD4 T cell engraftment with minimal toxicity. The same procedure followed by repletion with modified CD4 and CD8 T cells suggests an additional effect on viral control during TI in three subjects. Sangamo is currently expanding this cohort to evaluate these findings.

Jeffrey Murry [44] reported that while screening for LRAs, several compounds such as proteasome inhibitors, glycogen synthase kinase 3 (GSK3) inhibitors and cyanotriazoles, are identified to induce latency reversal in primary CD4 T cells infected *in vitro* with an HIV reporter virus. GS-46 (3.1-fold activation of supernatant virion production *ex vivo*) belongs to the cyanotriazoles and has been found to synergise with several proteasome inhibitors (bortezomib, carfilzomib, oprozomib) during treatment of resting CD4+ T cells from cART-suppressed HIV-positive participants. GS-46 and bortezomib in particular, present a magnitude and frequency of HIV induction *ex vivo* (40-fold activation) close to that of mitogen activators (63-fold induction), with minimal effects on cellular proliferation, T cell activation marker expression and cytokine production. Transcriptomic analysis shows that GS-46 broadly affects host gene expression but has no HDACi/HMTi (histone methyl transferase inhibitor) activity.

Alberto Bosque [45] closed this session by showing that a medium-throughput screen of chemical libraries allowed identification of a family of compounds (triazol-1-ol analogs) that trigger viral reactivation. In a cultured T_{CM} model of HIV latency, triazol-1-ol analogs and particularly HODHBt (3-hydroxy-1,2,3-benzotriazin-4(3H)-one) or HATU (O-(7-azabenzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium hexafluorophosphate) can reactivate latent HIV-1 in a dose-dependent manner. This result has also been confirmed after treatment of latently HIV-1-infected cells isolated from aviraemic patients with HOAt (1-hydroxy-7-azabenzotriazole) and latency reversal analysed using the QVOA assay or the modified REVEAL assay. The family of triazol-1-ol analogs reactivates latent HIV-1 without T cell proliferation, activation or cytokine release. Analysis of the mechanism of action involves downregulation of STAT5 activity.

Session 8: practical issues in designing HIV cure trials

Joe Eron [46] opened this session by listing the many challenges ahead in HIV cure research. He pointed out that barriers to cure are currently well established and include virus-expressing reservoirs, latently infected cells, as well as failure of HIV-specific immunity. However, methods for measuring the HIV reservoir may have very limited discriminating power and should be adapted to different trial endpoints based on intervention.

Combination 'kick and kill' interventions present many challenging issues, including study design questions, sample size and control arms according to outcomes, HIV-infected subpopulation studied (acute HIV infection or chronically infected individuals), surrogate markers, as well as the acceptable level of risk that could be taken by healthy HIV-infected volunteers under suppressive treatment.

According to Alison Hill [47] mathematical models could be a useful tool to help conduct cure trials in the face of uncertainty about drug efficacy and patient outcomes. Models have initially been used to interpret changes in viral dynamics seen with antiretroviral treatments. A new model has been developed to predict the effect of reservoir-reducing therapies in terms of type and time of viral rebound, as well as log-reduction in the reservoir size. Treatment interruption trials are currently necessary to estimate efficacy of LRAs, size of residual reservoir given time of rebound, number of patients needed to estimate LRA efficacy, length of follow-up and frequency of viral load sampling.

Jessica Salzwedel [48] emphasised the fact that stakeholder engagement can have a positive influence on the research process. This engagement is now part of the Good Participatory Practice (GPP) that includes normative guidance around stakeholders' role in research. A GPP framework is particularly important in HIV-prevention and HIV-cure trials. Developing comprehensive engagement plans that reflect sociocultural norms and stakeholder needs is crucial to shaping the research process, advancing research aims and ensuring successful trials.

Karine Dubé and Jeff Taylor [49] reported preliminary results of an extensive survey conducted in September–October 2015 among potential HIV cure research participants in the US. Attitudinal surveys among people living with HIV about cure research are rare. In the presented survey more than 50% of respondents would be willing to participate and around two-thirds of respondents were willing to interrupt treatment. Potential volunteers were more willing to participate in low-risk studies. Perceived potential personal 'benefits' include feeling good about contributing and helping to find a cure. Perceived clinical risks included possibility of developing resistance to ARVs, toxicities, and gene activation that could cause cancer. It could be helpful if community education about HIV cure was a part of scientific research programs.

Sessions 9 and 10: new therapeutic approaches

Elimination of the latent pool of infected cells that persists on cART, as well as the undefined cells that are the source of low-level viraemia observed in some treated patients, requires rationally designed strategies. Disruption of viral dormancy by LRAs to induce viral antigen expression in infected cells is under clinical investigation. Therefore, the need to control persistent inflammation and to enhance the ability to clear persistent infection by cytotoxicity and/or specific antibodies is also under investigation. Cure strategy should be able to clear all infected cells including those present in the germinal centre of lymphoid follicles and in immune privileged sites like the CNS and testes.

The goal for any cure should have the '3 S' characteristics: simple, safe, and scalable.

The highlights of the new therapeutic approaches to break viral persistence are reported herein.

John Mascola [50] presented on the role of neutralising antibodies (NAb) in HIV infection during the opening lecture. The presentation highlighted the clinical work by Caskey *et al.* [51] who showed that a single infusion of 3BNC117 broadly NAb binding CD4 molecules was safe and significantly reduced plasma viral load by 0.8–2.5 log in 28 days in untreated patients. However, emergence of resistant viral strains was observed in some patients, pointing out the need for antibody combinations to further control viral replication. More potent NAb generated by molecular engineering methods, having a significantly longer half-life, empowered to both harness antibody-dependent cell-mediated cytotoxicity and engage Fc receptors to enhance immune control are under development.

Jeffrey Nordstrom [52] from MacroGenics, reported on their pilot work on DART bispecific, antibody-derived molecules exhibiting an HIV-binding arm based on broad binding antibody targeting conserved regions of Env paired to a binding arm for CD3 T cells. The CD3-binding arm of the construct should activate CD8 cytotoxicity and latently infected cells as previously reported this year by Sung *et al.* [53] and Pegu *et al.* [54]. The HIVxCD3 DART molecules induced the killing of HIV-infected quiescent and activated cells *ex vivo*.

Julia Sung [55] published that DART proteins were able to clear infected cells upon exposure to vorinostat, a latency-reversing agent. The same group reported now on viral sequences in a patient in whom the kinetics of viral clearance by DARTs was delayed. Viral clearance assays and sequencing showed that DARTs were capable of redirecting T cells against resting T cells, in the absence of viral escape. It is likely that the combination of LRAs and DARTs may represent a new way to deplete the viral reservoir. However, additional studies will be needed to induce significant killing of infected cells *ex vivo* with such constructs.

Amanda Beatrix Macedo [56] presented results on a potent agonist of Toll-like receptor 2/7 as T cell immune activation to reverse viral latency. The synthetic multi-pattern recognition receptor (PRR) agonist CL572 was able to trigger activation of latent proviruses in primary cells. It will be important to show that CL572 on its own will be sufficient to sensitize reactivated T cells to the cytopathic effect of HIV.

Sara Morón-López [57] assessed the merit of a therapeutic T cell vaccine targeting a conserved region of the HIV proteome in order to enhance immune control. A Phase 1 study with 24 early cART-treated patients was conducted to assess the influence of ChAdV63 and MVA.HIV on latent reservoir dynamics. Response to conserved regions increased in all vaccinated subjects. However, levels and decay of proviral DNA after cART were not associated with vaccine-induced immunogenicity, nor were they different from non-vaccinated subjects.

Barbara Felber [58] reported on new DNA vaccine developed to overcome HIV sequence diversity and to enhance immune responses to epitopes linked to virus control (Gag). This team engineered DNA-based immunogens consisting of conserved elements, with broad HLA-coverage and induced viral control in macaques. Importantly, this novel vaccine elicited broad CTL responses also in the 'hard to reach' germinal centre and effector mucosa sites.

Kamel Khalili [59] used the newly developed 'genetic cutter', the RNA-guided CRISPR/Cas-based (clustered regularly interspaced short palindromic repeats) DNA cleavage, to 'surgically' remove the entire HIV genome spanning between the two LTRs of the integrated provirus. Such compulsory reactivation induced cellular suicide through toxic build-up of viral proteins within HIV-1 latent Jurkat T cells.

Luis Giavedoni [60] also presented on the same CRISPR/Cas technology with a focus on antiviral strategies that target nanoparticles to CD4 T cells for the delivery of SIV-specific RNA-guided Cas9 nucleases. A dramatic reduction of SIV p27 antigen was observed after a combined targeting of LTR, ribosome slip site and TAR regions, showing the potential for eliminated viral replication.

Importantly, gene editing using the CRISPR/Cas9 system can serve as a novel HIV latency-reversing therapeutic tool for the permanent elimination of HIV-1 latent reservoirs. The development of such technology that can manipulate several genes at the same time represents a promising tool. However, there is a need to assess off-target effects.

Rafick Sékaly [61] presented a comprehensive review on immune exhaustion and its influence on the size of HIV reservoir and its localisation in the different CD4 T cell subsets. From the McGill University leukapheresis cohort, he compared transcriptome profiling of infected CD4 T cells according to PD-1 or Lag-3 cellular expression. Significant differences in CD4 T cell subsets and in transcriptome expression were observed in the PD-1 or Lag-3^{high} expressing cells. Inflammation and homeostatic proliferation involving all memory T cells were linked with elevated PD-1 expression, while a distinctive metabolic profile for glucose, lipids and amino acids characterised Lag-3^{high} cells. Such study findings dictate that an immunometabolism-based strategy should be considered for eliminating latently infected cells.

Keynote lecture

The keynote lecture was given by Carl Dieffenbach, Director of the Division of AIDS at the NIAID. His talk was about the HIV cure agenda at NIAID. First, he pointed out that a cure must be simple, safe and scalable [62,63]. His lecture was divided into three sections.

In the first section, he spoke on the establishment of the HIV reservoir using the example of the Mississippi child, he described the evolution of the HIV infection and asked what are the critical steps and timing during the 24–48 hours after birth regarding the establishment of the reservoir and the immune response to HIV. He presented two further studies as examples of very early treatment: the IMPAACT study P1115 that tried very early intensive cART in HIV-infected infants and the TIES study where infants were treated early within 24–48 hours of birth.

In the second part of the keynote lecture, Dr Dieffenbach talked about characterising the HIV reservoir. He pointed out that there are anatomical reservoirs like B cell follicles from which CTL are excluded. 'The tissue is the issue', he said, 'we sample blood only for convenience'.

In the third part of the lecture, he discussed novel interventions and research opportunities. Dr Dieffenbach said that we need to have agents that both suppress virus replication and promote killing of the infected cells at all the sites of new and ongoing replication.

He listed a wide variety of possible ways:

- Genetic strategies, including zinc finger nucleases and CRISPR/Cas 9;
- Strategies targeting the state of cell activation: but do these methods of activation simply propagate the reservoir?
- Methods of putting the provirus permanently to sleep: antisense transcripts, Polycomb complex;
- Monoclonal antibodies;
- DARTs that direct T cell-mediated cytolysis of HIV latently infected cells;
- Chimeric antigen receptor technology (CARs);
- Therapeutic vaccines: but they have not really shown activity so far, except the CMV vector;
- Combining immunotherapy with cART.

Dr Dieffenbach then drew interesting parallels between the search for the HIV reservoir and the recently identified reservoirs of Ebola virus in the eye [64] and semen [65,66]. He also drew parallels between the current state of the HIV cure field and that of the HIV field in the mid-1980s, prior to the demonstration of efficacy in the earliest AZT monotherapy trials.

Finally, Dr Dieffenbach reminded everyone of the commitment of President Obama in the search towards an HIV cure.

Satellite symposium: NIAID Martin Delaney Collaboratories

A satellite symposium was convened by the National Institute of Allergy and Infectious Diseases (NIAID) to discuss progress and the challenges faced by the three Martin Delaney Collaboratories (MDC) for HIV cure research. The MDC program, begun in July 2011, is co-funded by NIAID and the National Institute of Mental Health (NIMH). The program was designed to facilitate partnerships between academia, industry, government and community to move

HIV cure research forwards more rapidly than could be accomplished by individual groups working alone.

The symposium consisted of updates from each of the three Collaboratories, focusing on research highlights, changes in personnel and structure, new collaborations formed, and plans for the remainder of the fifth and final year of funding. These updates were followed by three panel discussions on topics pertinent to each of the three Collaboratories, as well as to the broader HIV cure research community.

The first presentation was given by Keith Jerome, co-principal investigator of the defeatHIV Collaboratory based at the Fred Hutchinson Cancer Research Center (FHCRC) in Seattle, Washington. defeatHIV is focused on cell and gene therapy approaches to render the host immune system resistant to HIV infection and to inactivate latent HIV provirus and thus eliminate the persistent HIV reservoir. Dr Jerome outlined the various endonucleases being pursued to target and deactivate HIV proviruses that are integrated into the genomes of latently infected cells, with particular focus on the HIV-targeted Mega-TAL enzymes developed by Andrew Scharenberg (FHCRC) that incorporate the binding region of a transcription activator-like effector nuclease (TALEN) with the cleavage sequence from a homing endonuclease [67]. These enzymes display superior specificity compared to the endonucleases alone, with virtually no off-target cleavage.

The second presentation was given by David Margolis, principal investigator of the CARE Collaboratory based at the University of North Carolina at Chapel Hill. Dr Margolis gave an overview of CARE's work to identify LRAs in collaboration with industry partner, Merck. CARE is testing 144 newly identified latency-reversing compounds with unknown mechanisms of action in five laboratories in a variety of primary cell models developed by CARE [68]. One novel class of LRA discovered so far is the farnesyltransferase inhibitors, which have little activity on their own, but synergise with histone deacetylase inhibitors to reactivate latent HIV. One candidate, MRK-17, demonstrated variable activity in resting memory T cells from four HIV-positive individuals.

The third and final MDC update presentation was given by Steven Deeks, co-principal investigator of the DARE Collaboratory based at the University of California, San Francisco. Dr Deeks highlighted the work that DARE has done to identify where the persistent viral reservoir resides within the body and how the immune system affects this reservoir. DARE and collaborators have zeroed in on B cell follicles within lymph nodes as a potential reservoir sanctuary in SIV controllers in the non-human primate model [69]. DARE plans to test a strategy to perturb the follicles to permit CD8 T cell infiltration and clearance of the CD4 T cell reservoir.

The first panel discussion was on the topic of 'Industry Partnerships and Overcoming Barriers to Translational Research'. Panel members expressed concerns that fewer pharmaceutical companies remain focused on HIV than in the past, and even fewer are actively engaged in cure research. One barrier seems to be the lack of validated biological targets, and another seems to be the lack of validated assays to measure reductions in the size of the replication-competent reservoir. Another challenge is that industry is reluctant to test drugs and biologics that are in clinical development for other therapeutic areas, such as cancer, in HIV-positive individuals because of the safety risk in a different patient population. One workaround is to carry out preclinical studies with 'tool' compounds instead. These are analogs of the drug candidate with similar function, but different chemical properties.

The second panel discussion was on 'ARVs and Optimizing Animal Models for Evaluation of HIV Cure Strategies'. Suppressing viraemia in non-human primates to enable cure studies requires large amounts of ARV drugs, which has been a significant hurdle for the field. Access to ARVs has improved significantly in the last couple of years, thanks to faster internal company processes, but access to large amounts of experimental agents to test in curative strategies remains a hurdle. In terms of choice of experimental model, the panel agreed that the model should be selected based on the scientific question to be asked, and that there is no 'standard' model appropriate for all indications. While, humanised mice allow *in vivo* testing against HIV in human cells, require less drug and enable quicker experimental turnaround than non-human primates, they also yield smaller amounts of blood and tissue, which can stymie efforts to measure reservoir endpoints, and drug toxicity is sometimes a limiting factor.

The third and final panel discussion was on 'Clinical Trial Design: Appropriate Populations and Endpoints for Control versus Eradication Studies'. Panel members discussed the challenges of measuring changes in the size of the reservoir in individuals who were treated early after infection because of the already very small initial size of their reservoir. Measuring reservoirs in individuals who were chronically infected prior to initiating therapy is easier due to the larger reservoir size, but their reservoirs may be more difficult to eradicate due to increased viral heterogeneity as compared to early-treated individuals. Some strategies may lead to control of viral rebound without necessarily reducing the reservoir size. Testing of such strategies will ultimately require treatment cessation. Engaging community and diverse, global populations will be critical to maintain transparency and to inform study design as the field evolves.

Conclusion

The 7th International Workshop on HIV Persistence during Therapy was an occasion for fruitful scientific exchanges. This report covers only the oral sessions but the poster presentations can be found in the abstract book (www.viruseradication.com). We will progressively post on www.hiv-persistence.com the PDF version of these posters as we get them from the authors.

The 8th International Workshop on HIV Persistence during Therapy will again be held in Miami, Florida on 12–15 December 2017 – save the date for your diaries!

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Conflict of interests

All authors declare no conflict of interests.

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